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COMPARISON OF POTENTIAL TOXIGENICITY OF *FUSARIUM SPP.* FROM THAILAND AND NORTH AMERICA

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Abstract

Three species of Fusarium *isolated from samples obtained from Thailand close to the Thai-Kampuchean border, and suspected trichothecenes producing fungi were* in vivo *compared their ability to produce trichothecenes (T-2, HT-2). The organisms were cultured on rye at 15 to 25° C for 24 days. The mouldy samples and autoclaved rye control were extracted with ethyl acetate and a part of each extract was cleaned up by thin layer chromatography and used for rabbit skin bioassay. The oily portion of the extract was reduced by hexane-methanol and the methanol fraction was used for animal inoculation and chemical analysis using gas chromatography and mass spectrometry. Each group of 5 mice was intragastrically inoculated with propylene glycol containing the clean-up sample. The results of skin bioassay, chemical analysis and mouse inoculation were agreeable. None of the three species from Thailand were found to produce neither T-2 toxin nor HT-2. In contrast,* F. sporotrichioides *from North American and American type of culture collection produced copious amounts of them.*

Introduction

Several species of the *Fusarium* fungi can produce mycotoxins particularly trichothecenes. These toxins have been associated with several types of mycotoxicoses in both animal and human[1] and it has been alleged that such mycotoxins were used in Southeast Asia as chemical warfare agent[2]. Yellow spots were reported to occur on trees and roofs in Thailand, 8 kms. from the Kampuchean border, on Febuary 19, 1982[2]. Samples of these spots were intensively studied a few days later[3]. The spots yielded pollen of plants, hyphae and spores of fungi. *Fusarium semitectum* var. *semitectum* and an isolate of *Fusarium* sp.[3] later identified as *F. moniliformis*[2] were cultivated from the spots. *F. semitectum* and *F. solini* were also isolated from a wilting banana shoot and from a leaf, collected around the area. Because it is unknown at this time whether *Fusarium* spp. from Southeast Asia are naturally capable of producing trichothecenes, these isolates were tested for their potential to produce toxins.

Materials and Methods

F. monoliformis (# 21982), isolated from a yellow spot on a leaf, and *F. solani* (DAOM 190 352), isolated from the surface not the yellow spot, of another leaf collected after the Febuary 1982 in Thailand, and *F. semitectum* (S03 - 5), isolated from a wilting banana shoot were used. For comparison, *F. sporotrichioides* (MCH 7452) isolated from mouldy hay in Saskatchewan, Canada[4], *F. sporotrichoides* ATCC 48018 and ATCC 48020 were used.

Each organism was cultured on autoclaved rye in 6 flasks (2 liters) containing an equal volume of grain and water (250 ml). The cultures were incubated at 24 days at various temperatures (Table 1 and 2) in the dark as previously described[5]. As controls, an autoclaved rye sample was used. The materials were air-dried and ground to fine powder.

Fifty grams of each ground sample were extracted with 2 x 100 ml volumes of ethyl acetate. After flash evaporation, the residue was dissolved in 1 ml of ethyl acetate and purified by thin-layer chromatography using T-2 toxin as a standard, and then eluted with chloroform methanol (9 : 1) twice. Fractions were flash-evaporated and dissolved in 3 ml ethyl acetate. These cleaned-up samples were used only for skin bioassay. Two microliters of the cleaned-up samples were applied topically to shaved rabbit skin, together with equal amount of serial dilutions of standard T-2 toxin (Sigma) from 1 μg/ml to 20 μg/ml[6]. The reactions were scored at 24 hours by comparing those of the test samples with the reactions of the T-2 toxin standards.

Approximately 500 g of each ground sample were also extracted as described above, but the extracts were fully cleaned-up with hexane-methanol to reduce the oily constituents. The methanol fraction was evaporated to near dryness and suspended in 3 ml of ethyl acetate. One ml of this stock solution was sent to Dr. R. Greenhalgh, Chemistry and Research Institute, Agriculture Canada, Ottawa, for chemical analysis, employing gas-chromatography with electron capture and mass spectrometric detector after prior derivatization with N-heptafluoro-butyrylimidazole[7]. T-2 toxin and HT-2 toxin were used as standards. The remainder was allowed to air-dry and then reconstituted with 3 ml of propylene glycol for use in animal studies. Each sample derived from *F. monoliforme, F. semitectum, F. solini* and *F. sporotrichioides* MCH 7452, and from autoclaved rye (control) were given to 5 mice (CD-1, Charles River, Canada Ltd., Laprairie, Que., weighing approximately 20-22 g) intragastrically, at a dose of 0.1 ml/10 g body weight). All dead mice were necropsied as soon as possible after death, the remainder were killed at 48 h. Various tissues were collected, fixed in formalin, processed routinely to obtain Hematoxylin-Eosin stained sections, and examined microscopically.

Results and Discussion

All mice given the rye extract (control), the extracts derived from *F. moniliforme, F. semitectum* and *F. solani* survived until killing at 48 hours, but the 5 mice given the

extract derived from *F. sporotrichioides* MCH 7452 died within 18 h (Table 1 and 2). There were no pathological changes in the mice given the extracts from *F. moniliformis* #21982 or *F. semitectum* S03-5 as well as the sample derived from autoclaved rye control but the 5 mice given the sample derived from *F. solani* had tubular nephrosis and 3 out of 5 had vacuolar degeneration and lipidosis in the liver. The mice derived from *F. sporotrichioides* MCH 7452 showed marked necrosis of lymphoid follicles of the spleen, necrosis of glomeruli and tubules of the kidneys and complete necrosis of the intestinal mucosa. The thymus glands had necrosis of the cortex, and 3 of 5 mice showed pynknotic changes in the lungs. These pathological changes were in agreement with trichothecene mycotoxicosis in various kinds of animal, particularly the damages of the gastrointestinal tract[8, 9] and the lymphoid system[10-12].

Skin irritations have been described for detecting trichothecenes including T-2 toxin[6], diacetoxyscirpenol, fusarinon-X, nivalenol[13] and crude extracts of *F. poae* and *F. sporotrichioides*[14, 15]. The results of skin bioassays and chemical analysis (Table 1 and 2) correspond fairly well to the pathological findings in these experiments.

None of the 3 isolates of *Fusarium* from Thailand were found to be able to produce T-2 toxin, a known naturally occurring trichothecene, but T-2 toxin was found from all samples derived from the 3 isolates of *Fusarium sporotrichioides* under the same laboratory conditions (rye medium, dark, 15-25°C). Although this did not mean that the Thai *Fusarium* are not able to produce T-2 toxin in nature, but the evidence possibly indicates that at 15-25°C which is the temperature range in the crop season of the Eastern and Northeastern parts of Thailand, they may not naturally produce T-2 toxin. This question should be further investigated since *Fusarium* spp. have a worldwide distribution, including Thailand and Southeast Asia.

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TABLE 1. RESULTS OF SKIN BIOASSAY, CHEMICAL ANALYSIS AND MOUSE INOCULATION OF MOULDY SAMPLES DERIVED FROM *FUSARIUM* SPP. ISOLATED FROM THAILAND.

Fungus/Source	Culture Temperature (°C)	Chemical analysis for T-2, HT-2*	Results of skin bioassay and *in vivo* study (mouse test)
F. moniliforme Sheld. (21982 ; isolated from yellow spot on leaf)	25°	negative	negative on skin test ; no lesions in mice
F. semitectum Berk. & Rav. (S03-5 ; isolated from a banana shoot)	25°	negative	negative on skin test ; no lesions in mice
as above (S03-5)	15°	negative	negative on skin test
as above (S03-5)	25°	negative	negative on skin test ; no lesions in mice
F. solani (Mart.) Sacc. (DAOM 190 352; isolated from a leaf, beside a yellow spot)	20°	negative	negative on skin test ; tubulonephrosis and hepatic lipidosis in mice
Autoclaved rye (control)	25°	negative	negative on skin test ; no lesions in mice

* Detection limit<0.05 ppm T-2.

TABLE 2. RESULTS OF SKIN BIOASSAY, CHEMICAL ANALYSIS AND MOUSE INOCULATION OF MOULDY SAMPLES DERIVED FROM SUSPECTED TRICHOTHECENE PRODUCING FUNGI.

Fungus/Source	Culture temperature (° C)	Chemical analysis for T-2, HT-2*	Results of skin bioassay and *in vivo* study (mouse test)
F. sporotrichioides Sherb. (MCH 7452 ; isolated from mouldy hay in Saskatchewan)	25°	10 ppm HT-2 40 ppm T-2	skin test : suggestive of 20 ppm T-2; all mice died within 18 h with necrosis of thymus, spleen, intestinal mucosa, kidney tubules and glomeruli and lungs
F. sporotrichioides (ATCC 48018)	15°	0.5 ppm HT-2 5.4 ppm T-2	skin test : suggestive of 2.5 ppm T-2
as above (48018)	20°	1.7 ppm HT-2 7.0 ppm T-2	skin test : suggestive of 2.5 ppm T-2
as above (48018)	25°	1.3 ppm HT-2 11.2 ppm T-2	skin test : suggestive of 2.5 ppm T-2
F. sporotrichioides (ATCC 48020)	15°	10 ppm HT-2 21 ppm T-2	skin test : suggestive of 20 ppm T-2
as above (48020)	20°	5 ppm HT-2 15 ppm T-2	skin test : suggestive of 15 ppm T-2
as above (48020)	25°	10 ppm HT-2 36 ppm T-2	skin test : suggestive of 10 ppm T-2

* Detection limit<0.05 ppm T-2.

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บทคัดย่อ

ได้ทำการทดลองเพาะเชื้อในตระกูล *Fusarium* ที่แยกได้จากประเทศไทย, ที่แยกได้จากประเทศคานาดา และเชื้อของ American Type of Culture Collection (ATCC) ในข้าวไรย์ ที่อุณหภูมิ 15 - 25°ซ นาน 24 วัน เพื่อเปรียบเทียบการสร้างสารพิษ trichothecenes ทำการทดสอบโดยวิธี rabbit skin bioassay โดยใช้ T-2 toxin เป็นสารพิษมาตรฐาน, ทดสอบทางเคมีโดยใช้ gas chromatography และ mass spectrophotometry มี T-2 toxin และ HT-2 toxin เป็นสารมาตรฐาน และยังได้ป้อนสารสกัดเข้าไปในกระเพาะของหนูถีบจักร 5 ตัวต่อสาร 1 ตัวอย่าง ใช้ข้าวไรย์ที่อบไอน้ำเป็นตัวควบคุมการทดสอบ ผลปรากฏว่า เชื้อ *Fusarium* ทั้ง 3 species จากประเทศไทยไม่ให้สารพิษ T-2 และ HT-2 เลย ในขณะที่ *F. sporotrichioides* ที่แยกได้จากคานาดา และของ ATCC ให้สารพิษไตรโคทีซีนส์ T-2 toxin และ/หรือ HT - 2 ทั้ง 3 ตัว